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US ARMY MEDICAL RESEARCH LABORATORY

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REPORT NO. 744

IMMUNIZATION STUDIES WITH NAJA NAJA VENOM DETOXIFIED BY PHOTOOXIDATION

(Final Report)

by
Walter F. Kocholaty, Ph.D.
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and
Billy D. Ashley, M.S.

30 June 1967

UNITED STATES ARMY
MEDICAL RESEARCH AND DEVELOPMENT COMMAND

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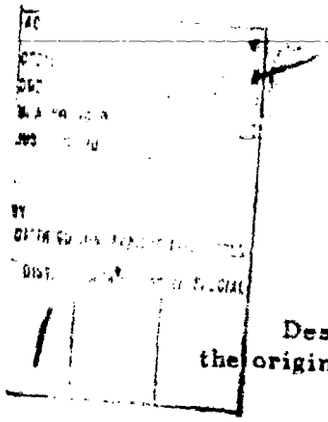

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Production of Polyvalent Antivenins
Work Unit No. 149
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ABSTRACT

IMMUNIZATION STUDIES WITH NAJA NAJA VENOM DETOXIFIED BY PHOTOOXIDATION

OBJECTIVE

The object was to detoxify cobra venom by photooxidation in such a way that the immunogenic properties of this venom were preserved.

RESULTS

Photooxidation of cobra venom by visible light in the presence of methylene blue resulted in a relatively slow detoxification of the venom. The detoxified venom when injected in rabbits caused a very weak immunogenic response.

CONCLUSION

The phenomenon of photooxidation in the detoxification of venoms appears to be generally applicable; however, the conditions for the immunogenic response may vary from venom to venom.

IMMUNIZATION STUDIES WITH NAJA NAJA VENOM DETOXIFIED BY PHOTOOXIDATION

INTRODUCTION

Detoxification of snake venoms by photooxidation with visible light in the presence of methylene blue was demonstrated first on Crotalus atrox venom (1) and extended to other venoms (2). Three of the photooxidized venoms tested for their immunogenic properties gave rise in rabbits to antibodies which protected mice from the toxic effects of these venoms. In an attempt to apply similar techniques to Naja naja venom some differences and difficulties were encountered which necessitated more detailed investigations reported herewith.

EXPERIMENTAL PROCEDURE

The venom was collected at the snake colony of this laboratory under ice, quick-frozen and lyophilized. The pooled collections were ground and sieved as described previously (1,2) and stored at 4 °C in a desiccator in the dark. The protein content of the centrifuged and filtered (Millipore Filter of 0.45 μm pore size) venom solution was 82.5% (biuret). Phospholipase A activity was determined as previously described (3). No proteolytic activity against casein could be demonstrated and esterase activity against TAME (p-toluene sulfonyl-L-arginine methyl ester) was absent. Photooxidation was carried out at pH 4.0 and 8.5. Toxicity and qualitative precipitin tests were conducted as described previously (2). The LD_{50} for this particular batch of venom was 7.5 γ I.V. and 8 γ I.P. (20-25 g mouse).

RESULTS

Effect of Photooxidation on Loss of Toxicity and Phospholipase A Activity. Table 1 summarizes the rapid loss of phospholipase A activity and concomitant loss in venom toxicity with increasing O_2 uptake and time of photooxidation.

Immunization of Rabbits and Mice with Photooxidized Venom of Naja naja. Venom of N. naja was photooxidized to an uptake of 20 μl O_2/mg protein using the reaction mixture and conditions described in Table 1 and stored in ice and in the dark. Prior to injections the photooxidized venom was diluted with physiological saline and mixed with an equal amount of Algivant (Colab Laboratories, Inc.). The weekly

immunization schedule for rabbits and mice was identical. Each animal received three injections a week (Monday, Wednesday, Friday), mice for 3 and rabbits for 4 weeks consecutively. The first two doses for mice (mature white Swiss, 30 to 40 g) were 32γ of photooxidized venom and the rest 64 γ administered subcutaneously in a volume of 0.1 ml for a total of 512γ.

Rabbits (white New Zealand) weighing 2-3 kg, received 1 mg venom protein in 1.0 ml for the first two injections and 2 mg in 1.6 ml for all subsequent injections for a total of 22 mg per animal. Injections were administered intramuscularly and alternated between the two hind legs. After a rest period of 1 week, mice were challenged with unaltered venom and rabbits were bled to death by heart puncture.

Neutralization and Protection Studies. When the mice which had been immunized with the photooxidized N. naja venom were challenged intravenously with increasing amounts of unaltered venom, all mice died within 24 hours even with the lowest dose (2 LD_{50}) employed.

Of the 10 rabbits (23-31, 33) immunized with photooxidized venom (Table 2), four did not show a positive (qualitative) percipitin test with the highest concentration of venom used, and one was positive only to the fourth dilution. The rest were weakly positive up to the sixth dilution.

The sera of the rabbits showing the highest (#23, 24, 25, 31, 33 = "A") and the lowest (#26-30 = "B") titer were combined and their γ-globulins isolated separately (4).

The neutralization and protection studies were carried out on mice using the γ-globulin obtained from rabbits of group A. As shown in Table 3, the globulins were practically inert in the neutralization as well as in the protection studies.

Since the results of the neutralization and protection studies were disappointing, five more rabbits were immunized with twice the amount of photooxidized venom for a total of 44 mg using the same adjuvant and an identical immunization and bleeding schedule as before. No lesions of significance were recognized in the tissues of all animals injected with N. naja venom. This information was furnished by Colonel M. A. Ross, VC, formerly Director, Pathology Division, USAMRL. One rabbit died during this period from causes not related to the immunization procedure.

All the rabbits (#32, 34, 37, 38 = "C") immunized with the higher amounts (44 mg total) of photooxidized venom responded with a positive though somewhat erratic precipitin test (Table 2). The γ-globulins were isolated as described before. The results shown in Table 3 are almost identical with the ones obtained in the previous experiment, where only one half as much photooxidized venom had been used in the immunization schedule.

Photooxidation of Naja naja Venom at Acidic pH and at Low Temperature. The comparatively low protection obtained with the rabbit immune globulins prompted a more detailed investigation into the various parameters of the photooxidation mechanism of the particular venom. Photooxidation may be carried out in a more selective manner utilizing an acidic reaction thus excluding histidine and tyrosine from photooxidative destruction (5, 6). Another specific variation may be introduced by working at temperatures below 37° (7).

Examination of the effect of pH on detoxification by photooxidation of this venom revealed the usual pattern with a pH optimum at about 8.5* and a steep drop toward the acid side, similar to that demonstrated with Crotalus atrox (1).

Studies of other parameters revealed that apparently the only factor related to the decrease in toxicity is the amount of O_2 uptake per mg venom protein irrespective of the concentration of the venom, the length of time of photooxidation, the pH of the reaction mixture (8.5 or 4.0), and the temperature (37° or 10° C). No significant volumes of CO_2 were developed during any of the variations in the photooxidation procedure. A summary of these experiments is illustrated in Figure 1.

DISCUSSION

The photooxidation of proteins by visible light in the presence of methylene blue permits gradual and subtle alterations of certain amino acid side-chains, which can be further modified by carrying out the reaction varying the pH and the temperature. The changes resulting in the molecule affected have provided an insight into functional groups of enzymes and a method for the detoxification of toxins and venoms(8). The latter has proven useful in providing modified venoms capable of

* At pH 9.0 and a concentration of 10 mg venom protein per ml a precipitate forms very rapidly in the initial stages of photooxidation.

eliciting antibodies in experimental animals with simultaneous abolishment of the necrogenic and hemolytic properties.

Why immunization with the photooxidized venom failed to produce an immunoglobulin capable of protecting mice is not understood at the present time. Attempts to vary the conditions for photooxidation (Fig. 1) revealed nothing of relevance to conduct photooxidation in a more promising way, or to indicate abnormalities in the chemical nature of this venom.

One may, however, speculate on two facets which stand out by contrast and, although not explainable at this time, may have a bearing on the failure to produce an immunoglobulin.

One, is the observation that the photooxidative detoxification of Naja naja venom proceeds at a considerably slower rate and with a high O_2 consumption compared with the other venoms investigated. From this one might suspect a unique arrangement of the four amino acids susceptible to photooxidation. The isolation of cobrotoxin, one of the toxic proteins from Formosan cobra venom, was reported recently by Yang (9). The amino acid composition of this compound has not yet been reported. Its molecular weight is 11,000. In this connection it is of interest to compare the rapid loss of phospholipase A activity with the very slow decrease in toxicity, a fact that would tend to indicate that this enzyme is, at least in its native form, a minor factor in the toxicity of this venom.

Two, is the observation of De Vries and his group (10) that the Vipera palestinae neurotoxin by itself is weakly antigenic, but when combined with carboxymethyl cellulose (CMC) the immunogenic properties are enhanced to such a degree that minute amounts of carboxymethyl cellulose-bound neurotoxin elicit a high antibody response. The Vipera palestinae neurotoxin recently isolated by these workers (11) has a low molecular weight (11,000) similar to that of the cobrotoxin of Yang.

De Vries and co-workers conclude that if a low molecular weight characterizes venom neurotoxins the increase in immunogenicity obtained by binding to carboxymethyl cellulose "may be a reflection of the increase in molecular weight of the antigen."

SUMMARY

Photooxidation of cobra venom by visible light in the presence of methylene blue resulted in a relatively slow detoxification of the venom.

The γ-globulin isolated from rabbits immunized with the detoxified venom gave poor protection in the animal experiments. Possible reasons for the weak response of the immune serum are discussed.

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TABLE 1

EFFECT OF PHOTOOXIDATION ON LOSS OF TOXICITY AND PHOSPHOLIPASE A ACTIVITY

% loss in phospholipase *A* activity	µl O_2 uptake/ mg venom protein	Time of photooxidation (min)	LD_{50} tolerated (20 g mouse)
69	5	10	2
93	10	29	4
100	20	111	8
-	27	258	16

Reaction mixture: 5 mg venom protein and 0.1 mg methylene blue in 0.1 M TRIS buffer, pH 8.5; total volume 2 ml; t = 37°C. No measurable CO_2 production was observed during the time of photooxidation.

TABLE 2

RESULTS OF THE QUALITATIVE PRECIPITIN TESTS OF SERA OF RABBITS IMMUNIZED WITH PHOTOOXIDIZED VENOM OF NAJA NAJA

Total amount of photooxidized venom injected	Rabbit No.	Tube No.* 1	2	3	4	5	6	7	8	9
22 mg	23, 24, 25	+	+	+	+	+	+	-	-	-
	26	+	+	+	+	-	-	-	-	-
	27, 28, 29, 30	-	-	-	-	-	-	-	-	-
	31, 33	+	+	+	+	+	+	-	-	-
	32	-	-	+	+	+	-	-	-	-
44 mg	34	+	-	+	+	-	-	-	±	-
	37	+	-	+	-	+	-	-	-	-
	38	+	±	±	+	+	+	-	±	-

*First tube contained 10 mg venom protein in 0.5 ml "dilute saline-borate buffer," pH 8.4 (4). All subsequent dilutions were 1:5. To each tube, two drops of serum were added. After 60 minutes at 37° C, the tubes were placed in the cold and readings were taken 24 hours later. Controls contained either saline and serum or saline and venom (10 mg) in amounts stated above. Both controls were negative.

TABLE 3

NEUTRALIZATION OF, AND PROTECTION AGAINST, Naja naja VENOM IN MICE BY RABBIT IMMUNE GLOBULIN

LD_{50}	Neutralization by Imm. Glob. "A"	Protection by Imm. Glob. "A"	Neutralization by Imm. Glob. "C"	Protection by Imm. Glob. "C"	Normal globulin control*	Venom control I. P.
1	0/3	3/3	0/3	0/4	1/4	5/8
2	0/3	3/3	0/4	0/4	4/4	8/8
3	3/3	3/3	1/4	2/4		
4	3/3	2/2	3/4	4/4		

Numbers are deaths over the total number of mice used. Readings were done 24 hours after injection.

Three or more mice received either 25 mg immune or normal rabbit globulin and the LD_{50} stated above in the neutralization tests.

Twenty-five mg of rabbit immune globulin was injected (I.V.) in a volume of 0.25 ml followed in 30 minutes by injection (I.P.) of 1-4 LD_{50} of Naja naja venom in the protection tests. Controls received venom only.

"A" Derived from rabbits (#23-25, 31, 33) immunized with a total of 22 mg photooxidized venom.

"C" Derived from rabbits (#32, 34, 37, 38) immunized with a total of 44 mg photooxidized venom.

* Rabbit γ-globulin, fraction II, purchased from Pentex Corporation.

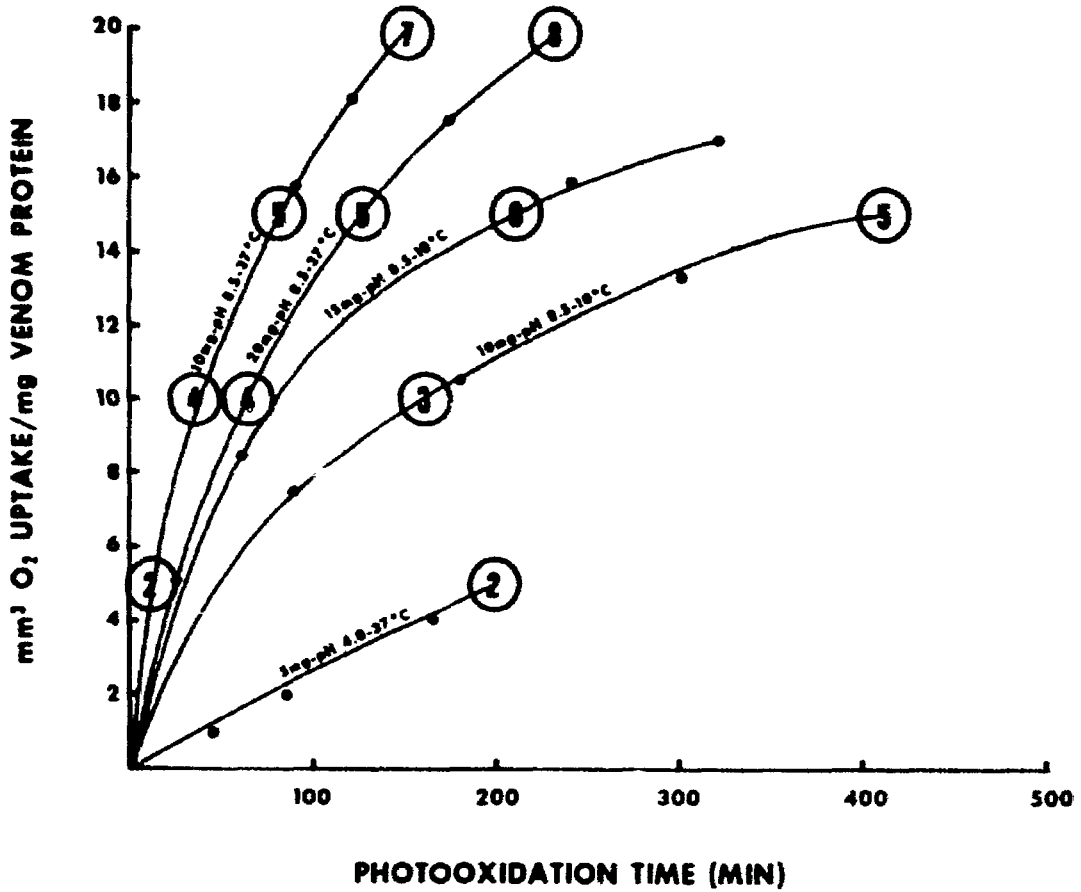

Fig. 1. Conditions for photooxidiation (pH temperature) are recorded on the O_2 uptake curves: mg refer to mg venom protein in 2 ml of reaction mixture. Numbers in squares indicate LD_{50} tolerated by 20 to 25 g mouse at the particular O_2 uptake.

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13. ABSTRACT

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